Biochimica et Biophysica Acta, 685 (1982) 273–278
Elsevier Biomedical Press

BBA 71064

Ca^{2+}-DEPENDENT K^+ TRANSPORT IN THE EHRLICH ASCITES TUMOR CELL

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(Received May 27th, 1981)
(Revised manuscript received September 28th, 1981)

Key words: Ca^{2+}-dependence; Ion channel; K^+ transport; Electron donor; Quinine; (Ehrlich ascites cell)

The possible presence and properties of the Ca^{2+}-dependent K^+ channel have been investigated in the Ehrlich ascites tumor cell. The treatment with ionophore A23187 + Ca^{2+}, propranolol or the electron donor system ascorbate-phenazine methosulphate, all of which activate that transport system in the human erythrocyte, produces in the Ehrlich cell a net loss of K^+ (balanced by the uptake of Na^+) and a stimulation of both the influx and the efflux of ^{86}Rb. These effects were antagonized by quinine, a known inhibitor of the Ca^{2+}-dependent K^+ channel in other cell systems, and by the addition of EGTA to the incubation medium. Ouabain did not have an inhibitory effect. These results suggests that the Ehrlich cell posseses a Ca^{2+}-dependent K^+ channel whose characteristics are similar to those described in other cell systems.

It has been reported that propranolol stimulates the uptake of glycine by the Ehrlich cell, the effect being due to a membrane hyperpolarization arising from an increase of the membrane permeability to K^+ [1]. Since propranolol increases the K^+ permeability and produces membrane hyperpolarization in the human red cell by the activation of the so-called Ca^{2+}-dependent K^+ channel [2,3], the same mechanism could explain the stimulation of amino acid transport observed in the Ehrlich cell.

The Ca^{2+}-dependent K^+ channel has been described in several tissues and cells [4]. It seems to play a role in the control of membrane potential of excitable tissues [5] and to be involved in the liberation of insulin by glucose from pancreas [6] and the release of K^+ by α-adrenergic drugs from the liver [7]. However, most detailed studies have been done in erythrocytes [8,9]. The system can be activated by an increase of cell Ca^{2+} [10], the effects of Ca^{2+} being modulated by poorly-known metabolic factors [11,12]. Reducing agents can activate the K^+ channel at subliminal Ca^{2+} concentrations [13]. The effects of Ca^{2+}, propranolol and reducing agents on K^+ transport are prevented by quinine [14,15].

In the present work the properties of the Ca^{2+}-dependent K^+ channel of the Ehrlich cell are investigated and compared to those observed in other cell preparations.

Materials and Methods

A tetraploid strain of Ehrlich ascites tumor cells, kindly provided by Dr. Y. Valladares from the Instituto Nacional de Oncología, Madrid, Spain, was used in all the experiments presented here. Experiments performed with a strain ob-

Abreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

tained from Dr. H.N. Christensen laboratory, University of Michigan, Ann Arbor, MI, U.S.A., gave similar results. Methods of collecting and handling the Ehrlich cell were as described by Inui and Christensen [16]. Except when stated otherwise, the experiments were carried out at room temperature, in air, and at a cell density of 5%. The standard incubation medium had the following composition (mM): NaCl, 135; KCl, 1; $MgSO_4$, 1.4; $CaCl_2$, 0.5; Hepes-NaOH, 20, pH 7.4. This low-K^+ medum was used in order to increase the sensitivity of the K^+-electrode measurements (see below). Incubation of the cells in this medium produced an initial loss of cell K^+ (10 to 30%), a steady concentration being reached after 30 min.

Net efflux of K^+ was estimated from the continuous measurement of its concentration in the medium with a K^+-sensitive electrode (Radiometer F2312K) [15]. The electrode was calibrated by adding known amounts of KCl to the incubation medium with and without cells. The loss of K^+ from cells measured with the electrode after complete permeabilization and equilibration in the presence of excess gramicidin D was consistent with the K^+ content of acid extracts of the same cell suspension measured by flame photometry.

Unidirectional fluxes of K^+ were estimated using ^{86}Rb as a tracer for K^+ [17,18]. For influx measurements, the incubation was terminated by 1:3 dilution with ice-cold 0.15 M choline chloride and centrifugation in the cold at 3000 rev./min for 1 min. The supernatant was removed [19] and the pellet was extracted with 1 ml of 3% sulphosalicylic acid. The radioactivity was determined in the extracts by Cerenkov counting, and the uptake of ^{86}Rb was finally expressed as the distibution ratio (cpm per ml of intracellular water/cpm per ml of incubation medium). Corrections for ^{86}Rb trapped in the extracellular space of the pellet were made from the uptake of ^{14}C-sucrose determined in parallel experiments. Na^+ and K^+ were determined by flame photometry and Cl^- by titration with $AgIO_3$ [20].

The efflux of ^{86}Rb was studied in cells loaded previously during 60 min at room temperature. Two experimental procedures were used to estimate the rate constant. In the first one, the loaded cells were washed and then incubated in medium containing no radioactivity; after different incubation periods, the cells were sedimented by rapid centrifugation (30 s at $10000 \times g$), and the supernatant solutions were saved and counted for radioactivity. The second procedure was a superfusion technique on Millipore filters: 5 mg of loaded cells were diluted to 5 ml and injected through a Swinnex 25 filter holder containing a glass fibre perfilter (AP25) and a 1.2 μm filter (RAWP). The cells were quickly washed with 10 ml of medium and then perfused at a rate of 2 ml/min, taking 1 ml fractions. At the end of the experiment the system was perfused with 10 ml of 6% trichloroacetic acid in order to recover the radioactivity remaining in the cells. ^{86}Rb was measured in each fraction by Cerenkov counting.

$^{86}RbCl$ and [^{14}C]sucrose were obtained from the Radiochemical Center, Amersham. Chemicals were purchased either from Sigma London or from E. Merck, Darmstadt. The ionophore A23187 was a generous gift from Elly Lilly Co.

Results

Fig. 1. shows that ionophore A23187, propranolol or the electron donor system, 20 mM sodium ascorbate + 0.1 mM phenazine methosulphate (ascorbate-phenazine methosulphate), caused a net loss of K^+ from the Ehrlich cell. The potassium loss was fast and transient with ionophore A23187 and slower and maintained with the other two agents. The shape of the time courses observed was similar to that found previously in guinea-pig hepatocytes (Ref. 7, and unpublished observations by Moreno, A. and Dominguez, C. in this laboratory). Quinine inhibited the loss of K^+, and removal of Ca^{2+} from the incubation medium (by adding excess EGTA) had similar effects (Fig. 1). Lanthanum chloride at 2 mM, another inhibitor of the Ca^{2+}-dependent K^+ transport in red cels [21], prevented also the K^+ loss induced by ionophore A23187 or propranolol, but this action was accompanied by agglutination of the cells (not shown). Noradrenalin and ATP, which cause K^+ loss in hepatocytes [7], and Pb^{2+}, which does so in the human erythrocyte [22], were also tested and found to have no measurable effect in the Ehrlich cell.

The activation of the Ca^{2+}-dependent K^+ channel promotes in the human erythrocyte a net

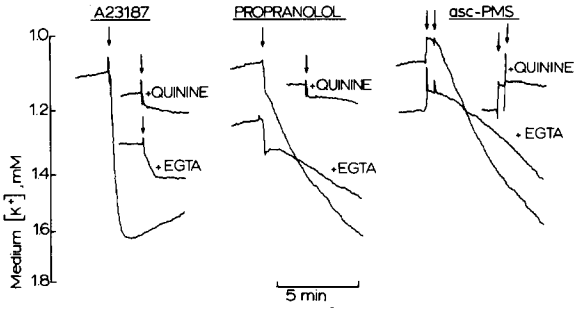

Fig. 1. Net loss of cell K^+ induced by ionophore A 23187 (2 μM), propranolol (0.5 mM) and ascorbate-phenazine methosulphate (asc-PMS), in the presence or in the absence of quinine (1 mM) or EGTA (3 mM). Cells were preincubated during 30 min at a cytocrit of 10%. Then they were diluted v/v with fresh medium. Quinine, when used, was added to that diluting medium, while EGTA was present during the whole preincubation period. The K^+ channel stimulators were added at the time marked with the arrows. The artefacts produced by the additions of propranolol and ascorbate appeared also in medium containing no cells and did not modify the response of the electrode to K^+. The apparent difference of medium K^+ at the beginning of the records in cells treated with quinine is also artefactual, whereas that observed in the experiments with EGTA corresponds to a real loss of cell K^+ (up to 6%) during the preincubation period. The cell K^+ levels at the end of the preincubation period ranged from 90 to 135 mequiv./kg of intracellular water. The increases of medium K^+ shown here correspond to losses of 12 to 18% of the total cell K^+ at the lowest point of the curves with no inhibitors.

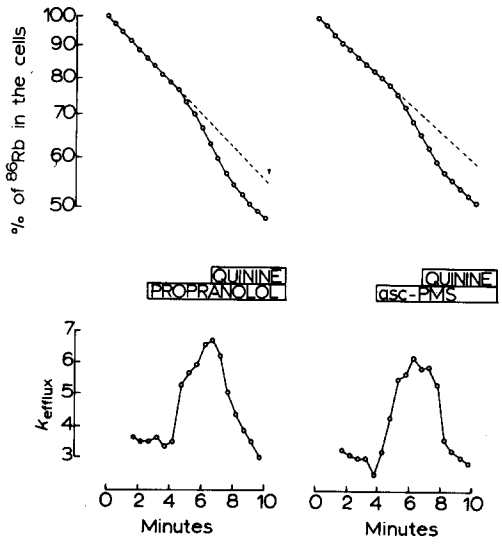

Fig. 2. Effects of propranolol and ascorbate-phenazine methosulfate (asc-PMS) on the efflux of ^{86}Rb. The experiments were performed using the Millipore superfusion technique (see Methods). In the upper part the changes of the ^{86}Rb contents of the cells (note the logarithmic scale) are shown. The lower part shows the changes in k_{efflux} (h^{-1}), calculated from the equation: $S_t/S_0 = e^{-kt}$.

loss of K^+ balanced by a net loss of Cl^-, with minor modifications of the Na^+ levels [15]. In contrast, the K^+ loss induced by propranolol in the Ehrlich cell was balanced mainly by the simultaneous uptake of Na^+ (Table I). This gain of Na^+ took place even when the external Na^+

TABLE I

EFFECTS OF PROPRANOLOL ON THE CELL LEVELS OF Na^+, K^+ and Cl^-

Cells were preincubated for 30 min in standard medium and then suspended in fresh medium with or without 0.5 mM propranolol and incubated for 15 min. Values are the mean ± S.E. of eight data and are expressed as mmol/kg of intracellular water.

	K^+	Na^+	Cl^-
Control	134±7	53±5	28±2
Propranolol	102±6	76±5	21±2
Net flux	−32	+23	−7

TABLE II

EFFECTS OF SEVERAL INHIBITORS ON THE EFLLUX OF ^{86}Rb INDUCED BY PROPRANOLOL

The efflux was measured during 15 min using the rapid centrifugation technique (see Methods). k_{efflux} was calculated as described in the legend to Fig. 2. Each value is the mean ± S.E. of four determinations.

Expt. No.	Circumstance	k_{efflux} (h^{-1})
1	Control	0.26±0.05
	20 mg/ml oligomycin	0.35±0.03
	0.5 mM propranolol	2.26±0.06
	+oligomycin	2.41±0.05
2	Control	0.51±0.06
	0.5 mM atebrin	0.38±0.02
	0.5 mM propranolol	2.22±0.04
	+atebrin	2.44±0.04
3	Control	0.27±0.01
	1 mM ouabain	0.33±0.02
	0.5 mM propranolol	2.42±0.08
	+ouabain	2.66±0.14

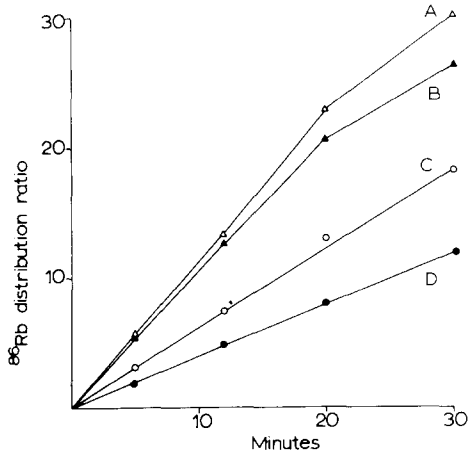

Fig. 3. Effects of propranolol on the uptake of ^{86}Rb. Additions: A, 0.5 mM propranolol; B, 0.5 mM propranolol + 1 mM ouabain; C, none; D, 1 mM ouabain. The cells were incubated for 30 min in standard medium prior to the beginning of the experiment.

concentration was kept below the intracellular one (data not shown), and could be hence interpreted as secondary to the membrane hyperpolarization induced by propranolol [1]. These differences in the ion that balances the K^+ loss are consistent with the fact that while the electrogenic permeability to Cl^- is much bigger than that to Na^+ in the human erythrocyte membrane [23] the reverse situation applies to the Ehrlich cell [24].

The results obtained with the K^+ electrode were further extended by measurements of unidirectional fluxes using $^{86}Rb^+$ as a tracer for K^+ [17,18]. Fig. 2. shows that the efflux of ^{86}Rb was

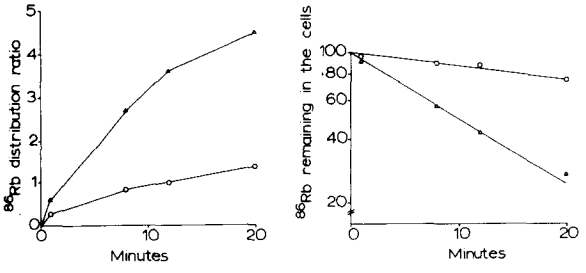

Fig. 4. Effects of ascorbate-phenazine methosulphate (asc-PMS) on the uptake (left) and on the efflux (right) of ^{86}Rb. ○——○, control; △——△, +asc-PMS. Both experiments were done in anaerobiosis using the same batch of cells subjected to identical preincubation conditions. Other details as in Fig. 3 and Table II.

stimulated by the treatment with propranolol or with ascorbate-phenazine methosulphate (see also Fig. 4 right), the effect of both of them being inhibited by quinine. The effects of other presumable inhibitors are shown in Table II. Ouabain had no effect indicating that the sodium pump is not involved. Oligomycin and atebrin, which are effective inhibitors in the erythrocyte [15,25], were without effect in the Ehrlich cell.

Fig. 3. shows that the uptake of ^{86}Rb was stimulated by propranolol and this stimulation was insensitive to ouabain. The effects of ascorbate-phenazine methosulphate on the uptake of ^{86}Rb were inconsistent from experiment to experiment when studied in aerobiosis. In three experiments a stimulation of the uptake (ranging from 41 to 93%) was observed, while in four experiments an inhibition (3 to 59%) was found. However, when the experiments were done in anaerobiosis, ascorbate-phenazine methosulphate always produced the expected stimulation of the uptake of ^{86}Rb (Fig. 4, left). These results suggest that the effects of ascorbate-phenazine methosulphate are, in the presence of O_2, not restricted to the Ca^{2+}-dependent K^+ channel.

Several other factors affected the response of the cells to the activators of the Ca^{2+}-dependent K^+ channel, although the results were not always reproducible. The incubation of the cells with no substrate before the experiment usually facilitated the loss of K^+ in response to ascorbate-phenazine methosulphate, while the preincubation of the cells with glucose during a 15–45 min period prevented to a variable degree the response to ascorbate-phenazine methosulphate or to propranolol. The interpretation of the last result was complicated by the fact that preincubation with glucose produced a net loss of cell K^+. Pershadsingh et al. [1] found that the hyperpolarization and the increase of amino acid uptake induced by propranolol was prevented in cells treated with rotenone, while the increase of ^{86}Rb efflux was not. In our hands, rotenone did not prevent either the K^+ loss induced by ascorbate-phenazine methosulphate or propranolol. The magnitude of the response to the different activators tested was quite variable from one experiment to another performed with a different batch of cells. Even though we have attempted to standarize the length and conditions of

the preincubation period we have been unable to identify the causes of such a variability.

Discussion

Our results point out that a Ca^{2+}-dependent K^+ channel is present in the plasma membrane of the Ehrlich cell. The characteristics of this channel regarding its activation by Ca^{2+}, propranolol or electron donors are similar to those described in erythrocytes [2,8,13]. The channel was insensitive to ouabain and inhibited by quinine, but other inhibitors that are effective in erythrocytes, such as atebrin and oligomycin, did not have an effect in the Ehrlich cell. The transient character of the effect of ionophore A23187 in the Ehrlich cell is not found in the erythrocytes [15], but it is reminiscent of the observations made in the hepatocytes [7]. The modifications in the rate of K^+ loss observed could well reflect transient modifications of the cytoplasmic Ca^{2+} concentration. It is reasonable to expect the effects of ionophore A23187 on the cytoplasmic Ca^{2+} levels in respiring cells to be more complicated than in the mammalian erythrocyte, since in those cells the ionophore acts presumably not only at the plasma membrane but also on the membranes of intracellular calcium stores [26]. Other particular properties of the Ehrlich cell such as the insensitivity to α-adrenergic drugs and to ATP could be due to the absence of the corresponding membrane receptors in the Ehrlich cell.

Although it has been proposed that the Ca^{2+}-dependent K^+ channel is under metabolic control in the red cell [11,12], this control seems more evident in the Ehrlich cell. Our observations on the changes of the sensitivity of the channel with the previous treatment of the cells point in this direction, but they do not indicate what are the metabolic changes involved.

Since Na^+-dependent transport is an electrogenic process [27–29], one could speculate that the Ca^{2+}-dependent K^+ channel could play a role in the regulation of that transport in the Ehrlich as well as in other cells. Thus, changes in the cytoplasmic Ca^{2+} concentration, the redox state of the cells or the extracellular levels of metabolites or hormones could act as control signals directing the energy stored in the transmembrane K^+ gradient towards the Na^+-dependent active transport systems through the activation of the Ca^{2+}-dependent K^+ channel and the subsequent changes of the transmembrane potential. The observation that propranolol leads to a stimulation of the uptake of glycine by the Ehrlich cell [1] is consistent with this view.

Acknowledgement

Financial support from the Fondo Nacional para la Investigación Científica is gratefully acknowledged.

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